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Food-Grade Cloning and Expression System for *Lactococcus lactis*

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A versatile set of cloning and expression vectors has been developed for application in self-cloning and other genetic modifications of *Lactococcus lactis*. The expression vectors were equipped with the controlled and strong *lacA* promoter of the lactococcal lactose operon. In addition, the transcriptional terminator of the aminopeptidase N gene, *pepN*, was inserted, which in some cases increased the genetic stabilities of the vectors and the cloned DNA. The small, 0.3-kb *lacF* gene encoding the soluble carrier enzyme IIA^{Lac} was used as a dominant selection marker in the plasmid-free *L. lactis* strain NZ3000 carrying an in-frame deletion of the chromosomal *lacF* gene. Lactose-utilizing transformants were easily selected on lactose indicator plates at high frequencies and showed a copy number of approximately 50 plasmids per cell. All vectors were stably maintained in the *lacF* strain NZ3000 when grown on lactose, and only the high-level expression vectors showed some instability when their host was grown on glucose-containing medium. The application potentials of the expression vectors carrying the *lacF* marker were determined by cloning of the promoterless *Escherichia coli gusA* reporter gene under control of the *lacA* promoter followed by analysis of its expression. While in one of the vectors this resulted in a promoter-down mutation in the −10 region of the *lacA* promoter, in other vectors high-level and controlled expression of the *gusA* gene was observed.

The development of a wide variety of cloning systems has allowed the improvement of many properties of *Lactococcus lactis* strains that are essential for a large number of industrial dairy and other food fermentations (10, 15). Those genetically improved lactococci and their products have great potential to be used in the food industry. However, the production strains used should be devoid of any antibiotic resistance markers that could compromise their applications in foods. As a consequence, vectors should contain selection markers that are acceptable in the food industry, and these are described here as food grade. Various food-grade systems have previously been proposed for *Lactococcus* spp. Some of these were based on homologous marker genes, such as the nisin resistance determinant *nsr* (14), while a heterologous system based on the sucrose utilization genes of *Pediococcus pentosaceus* has been designed (16). However, the application of these systems is limited to the cloning of a lactococcal bacteriophage resistance gene by using the *nsr* marker gene (14). The complementation of auxotrophic mutants is another approach to develop homologous markers that may allow for simple and dominant selection. Such a complementation system, based on nonsense suppressors of mutations in the lactococcal purine biosynthetic pathway, has recently been developed (12), while a marker system based on the *L. lactis* thymidylate synthase gene, *thyA*, has been proposed but not evaluated because of a lack of the appropriate mutants (19). The detailed characterization of the *L. lactis lac* operon encoding the lactose phosphotransferase system and tagatose-6-phosphate pathway (7, 8, 25, 26) has provided the possibility of developing a dominant homologous marker based on lactose complementation (6). The two elements of this system included the small 0.3-kb *lacF* gene coding for the soluble enzyme IIA^{Lac}, which was expressed by a vector-located promoter, and the lactose-deficient *L. lactis* strain YP2-5, which contained a missense mutation in the *lacF* gene (7). Evaluation of this first example of a homologous selection system for lactic acid bacteria showed its advantages, which included simple selection with lactose indicator plates and high stability during growth on lactose-containing industrial media (6). However, in order to fully exploit this marker system, there has been a need to obtain a series of useful cloning and expression vectors. Here we describe the development and application of stable and versatile vectors based on the high-copy-number endogenous *L. lactis* pSH71 replicon, the *lacF* selection marker, and the lactose-inducible *lacA* promoter.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The *L. lactis* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MC1061 was grown in Luria broth (20) at 37°C. *Lactococcus* strains were routinely grown at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) (23) supplemented with 0.5% lactose or glucose. The ability to ferment lactose was tested on indicator agar based on Elliker broth (13) containing 0.004% bromocresol purple and 0.5% lactose. Histochemical screening for *gusA*-positive clones was performed with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) at a final concentration of 0.5 mM. Chloramphenicol (10 μg/ml) and ampicillin (100 μg/ml) were used when appropriate.

Molecular cloning, reagents, and enzymes. Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method (1). Plasmid DNA from *L. lactis* was isolated by a modification of this procedure (8), and total DNA of *L. lactis* was isolated as described previously (10). Plasmid DNA was transformed into *L. lactis* by the method of Wells et al. (28). All other cloning procedures and *E. coli* manipulations were performed as described previously (20). Enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), New England BioLabs Inc. (Beverly, Mass.), or Boehringer GmbH (Mannheim, Germany) and used according to the instructions of the manufacturers. Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Biosearch, San Rafael, Calif.). *para*-Nitrophenyl-β-D-glucuronic acid was obtained from Clonetech Lab. Inc. (Palo Alto, Calif.).

The assay of β-glucuronidase activity to determine the promoter strength in the expression vectors with the *gusA* gene was as previously described (18).

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TABLE 1. Strains and plasmids used

Strain or plasmid	Relevant features	Reference or source
L. lactis MG5267	Lac$^+$, plasmid free, single chromosomal copy of *lac* operon	24
L. lactis NZ3000	Δ*lacF*, derived from MG5267 by replacement recombination	11
E. coli MC1061	*araD139 lacX74 galU galK hsr hsm$^+$ strA*	3
pUC19	Ap^r; cloning vector	27
pUC-T	Ap^r, T_{pepN}; 0.3-kb *Mlu*I-*Hin*dIII *pepN* terminator fragment joined to pUC19 *Hin*dIII-*Sal*I	This work
pNZ124	Cm^r; pSH71-derived lactococcal cloning vector	5, 18
pNZ272	Cm^r; *gusA* promoter-probe vector derived from pNZ124	18
pNZ1125	*lacF*; contains *pepN* gene	10
pNZ3004	Cm^r; pGKV210 derivative harboring the *lacA* promoter	24
pNZ2101	Cm^r, T_{pepN}; 0.3-kb *Hin*dIII-*Sal*I *pepN* terminator fragment from pUC-T inserted into *Xho*I-*Sal*I-digested pNZ124	This work
pNZ2102	Cm^r, P_{lacA}; 0.5-kb *Eco*RI-*Pst*I *lacA* promoter fragment of pNZ3004 inserted into *Pva*II-*Pst*I-digested pNZ124	This work
pNZ2103	Cm^r, P_{lacA}, T_{pepN}; 0.5-kb *Eco*RI-*Pst*I *lacA* promoter fragment of pNZ3004 inserted into *Pva*II-*Pst*I-digested pNZ2101	This work
pNZ2104	*lacF*; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ124	This work (Fig. 2)
pNZ2105	*lacF*, T_{pepN}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2101	This work (Fig. 2)
pNZ2106	*lacF*, P_{lacA}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2102	This work
pNZ2107	*lacF*, P_{lacA}, T_{pepN}; 0.4-kb *Nco*I-*Bam*HI *lacF* fragment of pNZ1125 inserted into *Sal*I-*Bgl*II-digested pNZ2103	This work
pNZ2120/pNZ2121	*lacF*, P_{lacA}; pNZ2106 derivative with a multiple cloning site	This work (Fig. 2)
pNZ2122/pNZ2123	*lacF*, P_{lacA}, T_{pepN}; 0.3-kb *Sst*I-*Nde*I *pepN* terminator fragment of pNZ2101 inserted into pNZ2120 or pNZ2121	This work (Fig. 2)
pNZ2116	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2102	This work
pNZ2118	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2106	This work
pNZ2119	*lacF*, P_{lacA}, T_{pepN}, *gusA*; 1.2-kb *Pst*I-*Hin*dIII *gusA* fragment of pNZ272 inserted into pNZ2107	This work

Vector constructions. The relevant properties of the constructed vectors are listed in Table 1. pUC-T was constructed by cloning the aminopeptidase N gene (*pepN*) terminator (22) as a 0.3-kb *Mlu*I (made blunt with Klenow DNA polymerase)-*Hin*dIII fragment in pUC19 (digested with *Hin*dIII and *Sal*I, with the latter made blunt with Klenow polymerase), using *E. coli* MC1061 as a host. The *pepN* terminator fragment was then isolated as a *Sal*I-*Hin*dIII fragment from pUC-T and cloned in the pSH71-based vector pNZ124 (18), which was digested with *Xho*I and *Hin*dIII. The resulting vector was designated pNZ2101. For the construction of vectors with high-level and controlled expression, the *L. lactis lacA* promoter was used to initiate transcription. The *lacA* promoter was isolated from plasmid pNZ3004 (24) digested with *Pst*I and *Eco*RI. The *Eco*RI site was made blunt with Klenow polymerase. The 0.5-kb fragment was cloned in vectors pNZ124 and pNZ2101 digested with *Pva*II and *Pst*I, resulting in pNZ2102 and pNZ2103, respectively. Vectors pNZ2101, pNZ2102, and pNZ2103 were constructed in *L. lactis* MG5267.

For the construction of homologous vectors from pNZ2101, pNZ2102, and pNZ2103, the chloramphenicol acetyltransferase gene was deleted by a restriction digestion with *Sal*I (made blunt with Klenow polymerase) and *Bgl*II and replaced with the *lacF* gene, isolated as a 0.4-kb *Nco*I-*Bam*HI fragment from plasmid pNZ1125 (the *Nco*I site was made blunt). Nonessential restriction sites were removed from plasmid pNZ2106, and a polylinker with multiple cloning sites was cloned upstream of the *lacA* promoter. Vectors pNZ2120 and pNZ2121 were constructed from plasmid pNZ2106 in four steps: (i) the small *Xba*I fragment was deleted; (ii) the resulting plasmid was digested with *Nde*I, made blunt with Klenow polymerase, and religated to remove this site; (iii) the restriction sites upstream of the *lacA* promoter were removed by an *Eco*RI-*Kpn*I digestion, the ends were filled in with Klenow polymerase, and the vector was religated; and (iv) finally, a synthetic double-stranded linker (5′-CTAGACAGCTGGGATCCGTCGACCCGGGCTGCAGGCATGCGGTACCACTAGTT-3′ and 5′-CTAGAACTAGTGGTACCGCATGCCTGCAGCCCGGGTCGACGGATCCCAGCTGT-3′) was cloned in the unique *Xba*I site, resulting in a multiple cloning site with unique restriction sites for *Pst*I, *Bam*HI, *Sal*I, *Sma*I, *Pst*I, *Sph*I, *Kpn*I, and *Spe*I. Vectors pNZ2122 and pNZ2123, which are derivatives of pNZ2107, were constructed by cloning the *Sst*I-*Nde*I fragment of pNZ2101 in the vectors pNZ2120 and pNZ2121 digested with the same enzymes. All of these vectors were constructed in *L. lactis* NZ3000.

The structures of the new plasmids that contained either the chloramphenicol resistance gene or the *lacF* gene as marker are summarized in Fig. 1 and were verified by single and double restriction enzyme digestions, while the orientations and sequences of the polylinkers carrying the multiple cloning sites were verified by nucleotide sequence analysis.

To test the application potentials of the vectors pNZ2102, pNZ2106, and pNZ2107, they were digested by *Pst*I and *Hin*dIII and used to clone the *E. coli gusA* gene, which was isolated from pNZ272 as a *Pst*I-*Hin*dIII fragment (18). *L. lactis* NZ3000 or the isogenic strain MG5267 was used as the host with ligation mixtures from either pNZ2106 and pNZ2107 or pNZ2102, respectively.

Determination of plasmid copy number per chromosome. Total DNA was digested with *Xba*I, separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane, and hybridized with a [γ-^{32}P]dATP-end-labeled *lacF* probe (5′-TTAATTACTTTGCTCCTCTGATCAC-3′). The hybridizing DNA fragments corresponding to the chromosomal and plasmid-encoded *lacF* genes were isolated, and the radioactivity was determined by using a liquid scintillation counter (LS7500; Beckman Instruments Inc., Palo Alto, Calif.). The ratio between the radioactivity obtained from the plasmid-located *lacF* gene and that obtained from the single chromosomal copy of *lacF* defines the copy number.

Nucleotide sequence analysis. The nucleotide sequences of the polylinkers in pNZ2110 and pNZ2111 and the *lacA* promoters in vectors pNZ2116, pNZ2118, and pNZ2119 were determined by the dideoxy-chain termination method (21), as modified by the AutoRead sequencing kit, and performed on the A.L.F. apparatus (Pharmacia Biotech, United Kingdom). A fluorescent primer specific for the *gusA* gene with the sequence 5′-GGGTTGGGGTTTCTACAGGACGTA-3′ was used (18).

RESULTS

Construction and characterization of food-grade cloning and expression vectors. To allow for the development of vectors carrying the *lacF* marker, a series of plasmids based on the lactococcal vector pNZ124 (18) was constructed; the plasmids have a common core structure consisting of the *L. lactis* promiscuous pSH71 replicon, the chloramphenicol resistance gene from the staphylococcal plasmid pC194, and a multiple cloning site (Fig. 1). Plasmids pNZ2102 and pNZ2103 were equipped with the controllable *L. lactis lacA* promoter (7, 24), while pNZ2101 and pNZ2103 additionally contain the *L. lactis pepN* terminator (22) (Table 1). These vectors were designed in such a way that a simple replacement cloning step would remove the chloramphenicol acetyltransferase gene and position the promoterless lactococcal *lacF* gene under control of the *repC* promoter (10) of the pSH71 replicon (Fig. 1). This resulted in the cloning vectors pNZ2104 and pNZ2105 (Fig. 2)

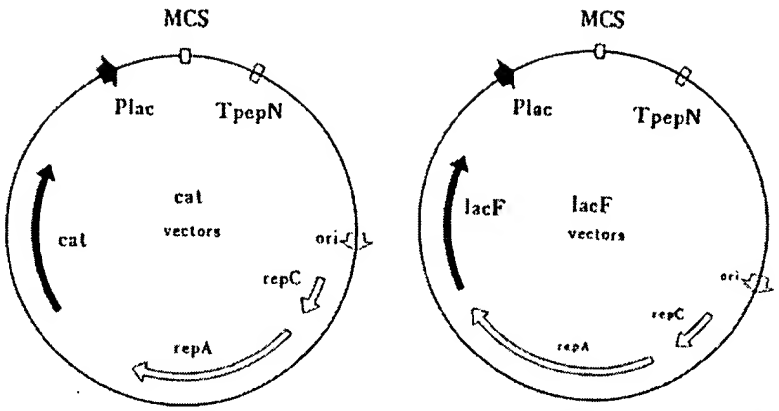

FIG. 1. Basic architecture of the constructed vectors carrying either a chloramphenicol resistance gene (left) or the *lacF* gene (right), which allows for food-grade selection. The minus origin of pS1171 (ori) is indicated, and the direction of the rolling-circle replication is indicated by the open arrow, which also indicates the direction of the *repC* promoter preceding the *repC* (repressor) and *repA* (replication protein) genes (10). MCS, multiple cloning site.

and the expression vectors pNZ2106 and pNZ2107, which harbor the *lacA* promoter without or with the *pepN* terminator, respectively. The two expression vectors were further improved by replacing nonessential restriction sites by both orientations of a newly designed polylinker, resulting in the pNZ2120/pNZ2121 and pNZ2122/pNZ2123, all of which contain the *lacA* promoter in the same orientation as the *repC* promoter driving expression of the *lacF* gene (Fig. 2). The lactose-inducible *lacA* promoter is known to induce high-level expression of downstream cloned genes (24). The construction of these vectors was performed in *L. lactis* NZ3000 by direct selection for acid formation from lactose on lactose indicator agar. All of the vectors carrying the *lacF* gene could easily be selected by lactose complementation of *L. lactis* NZ3000, indicating that the *repC* promoter drives efficient expression of the *lacF* gene. This was also evident from the rapid growth to high densities on lactose-containing medium of *L. lactis* NZ3000 harboring either one of the vectors (data not shown).

Application of the new vectors. To evaluate the use of the new vectors in gene cloning and controlled expression, the promoterless *E. coli* β-glucuronidase (*gusA*) gene was cloned under control of the *lacA* promoter. A plasmid with the expected size and configuration as determined by restriction enzyme digestion was readily obtained with pNZ2102, and the presence of this plasmid, designated pNZ2116, in *L. lactis* MG5276 gave rise to blue colonies on X-Gluc plates. However, when the *gusA* gene was inserted under control of the *lacA* promoter in pNZ2103, which also contained the *pepN* terminator, unexpectedly only white transformants of MG5276 were obtained on X-Gluc plates. Plasmid DNAs were isolated from 10 transformants, and their restriction digestion patterns showed a similar-sized plasmid that was larger than expected and hence not used for further studies.

The *gusA* gene was subsequently inserted into the expression vectors pNZ2106 and pNZ2107 and transformed into the *lacF*-deficient strain NZ3000. Lactose-utilizing transformants were readily obtained with plasmid pNZ2107 and gave rise to blue colonies on plates containing X-Gluc. All transformants tested contained a plasmid with the expected configuration, and one of those was designated pNZ2119. However, cloning of the *gusA* gene in pNZ2106 resulted in only one blue colony out of 500 transformants. The plasmid of this blue transformant showed the expected configuration and was designated pNZ2118.

Control of *gusA* gene expression in the *lacA* expression plasmids. To study the regulation of the *lacA* promoter in the different *gusA*-containing plasmids, the β-glucuronidase activities in lysates of cells grown in glucose or lactose were compared (Table 2). Strain NZ3000 harboring pNZ2118 showed the lowest β-glucuronidase activity and demonstrated no regulation (see below). The highest β-glucuronidase activity was observed in *L. lactis* NZ3000 harboring the vector pNZ2119. In this strain the induction of *gusA* expression upon growth on lactose-containing medium is twofold higher than that on glucose, as was also the case for strain MG5276 harboring pNZ2116.

Copy number and stability determination. The copy numbers of the new cloning and expression vectors in strain NZ3000 were compared (Fig. 3). Vectors pNZ2105, pNZ2106, and pNZ2107 have approximately the same copy number, whereas plasmids pNZ2104 and pNZ2118 (overexpressing the *gusA* gene), remarkably, showed a higher copy number. In contrast, plasmid pNZ2119, which also contains the *gusA* gene, has a copy number in *L. lactis* that is considerably reduced compared with those of the other plasmids. The exact plasmid copy numbers were determined for the expression vectors pNZ2106 and pNZ2107 and were found to be 46 and 36 copies per chromosome, respectively. Although no efforts to specifically detect insertion of the *lacF*-carrying plasmids in the chromosomal *lacF* locus were made, no such integration was ever observed in the Southern blot analysis during these copy number determinations.

The segregational instabilities of the vectors with or without the *gusA* gene were examined under selective and nonselective conditions. This was tested by growing strain NZ3000 harboring one of the plasmids in M17 medium containing 0.5% glucose or lactose and then plating appropriate dilutions on lactose indicator plates. All lactose-utilizing colonies appeared to have retained the plasmid. All of the vectors were stably maintained for more than 100 generations on lactose-containing M17 medium, and single colonies obtained after this period of growth were found to harbor plasmids with the expected size and restriction pattern (data not shown). Most of the vectors were also stably maintained when cultured on M17 medium

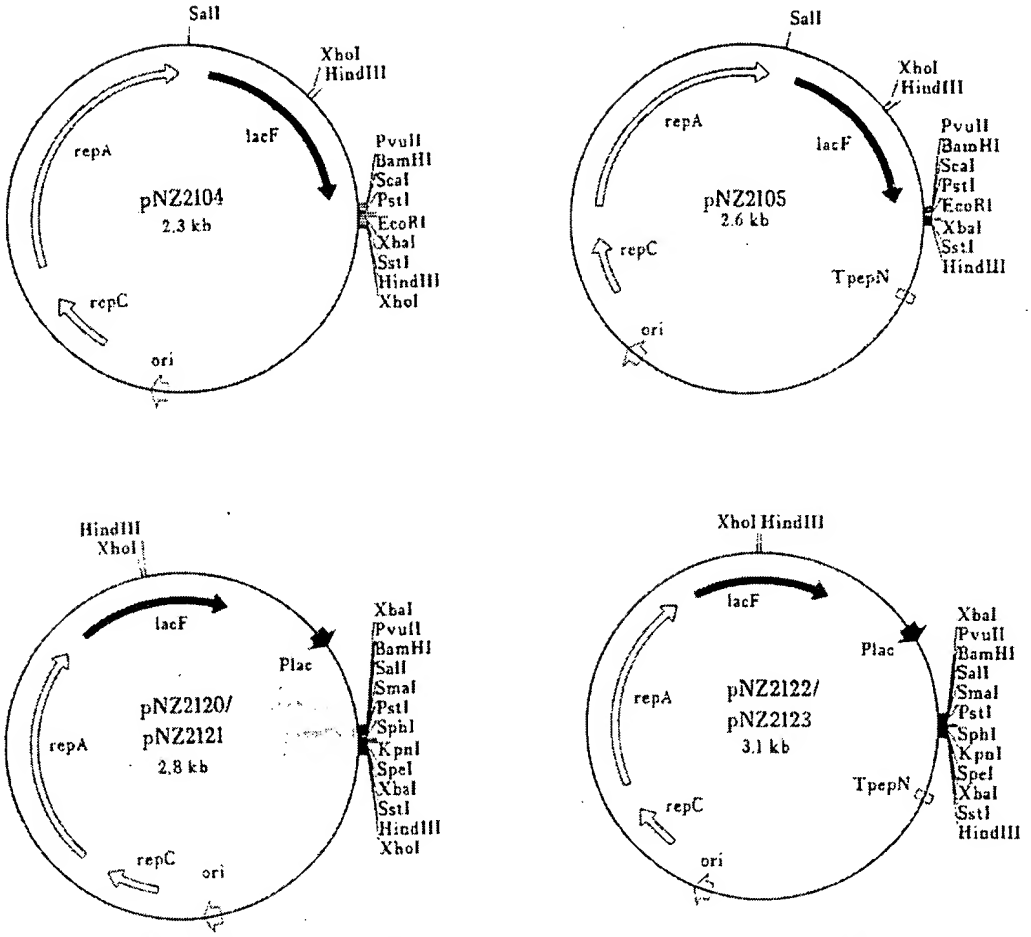

FIG. 2. Food-grade cloning and expression vectors. For explanations of symbols, see the legend to Fig. 1.

containing glucose (Table 3). However, when grown on glucose-M17, *L. lactis* NZ3000 harboring pNZ2118 or pNZ2106 showed a slightly reduced stability, while NZ3000 cells harboring pNZ2119 showed the highest segregational instability under these conditions.

Plasmid pNZ2118 contains a regulatory, promoter-down mutation in the *lacA* −10 region. To determine the origin of the variation in β-glucuronidase expression and to explain the observation that expression is not regulated in strain NZ3000

TABLE 2. β-Glucuronidase activities

Vector	β-Glucuronidase activity[a] (mean ± SD) of cells grown on:	
	Lactose	Glucose
pNZ2116	1,006 ± 128	586 ± 101
pNZ2118	367 ± 102	412 ± 137
pNZ2119	3,933 ± 486	2,043 ± 165

[a] Expressed as nanomoles per minute per milligram of protein. Data are for three independent determinations.

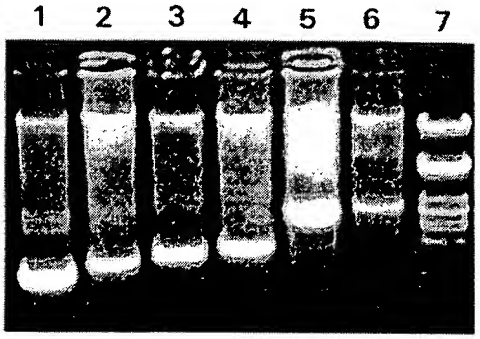

FIG. 3. Plasmid copy numbers of the food-grade cloning and expression vectors. Plasmid DNAs were extracted from equal amounts (based on the optical density of the culture) of NZ3000 cells harboring the different plasmids, digested with *Eco*RI, and separated by agarose gel electrophoresis. Lane 1, pNZ2104; lane 2, pNZ2105; lane 3, pNZ2106; lane 4, pNZ2107; lane 5, pNZ2118; lane 6, pNZ2119; lane 7, bacteriophage λ DNA digested with *Hin*dIII (marker).

TABLE 3. Stabilities of the food-grade vectors

Plasmid	Plasmid loss/generation
pNZ2104	6×10^{-4}
pNZ2105	1×10^{-4}
pNZ2106	2×10^{-3}
pNZ2107	9×10^{-4}
pNZ2118	3×10^{-3}
pNZ2119	1×10^{-2}

harboring pNZ2118, the nucleotide sequences of the *lacA* promoter in the *gusA*-containing plasmids pNZ2116, pNZ2118, and pNZ2119 were determined. Both pNZ2116 and pNZ2119 contained the bona fide *lacA* promoter (24). In contrast, the *lacA* promoter in plasmid pNZ2118 contained a single mutation at position −11, where a T residue was replaced by a C residue (Fig. 4).

DISCUSSION

To allow for further exploitation of the food-grade marker system based on the lactococcal *lacF* complementation system (7), we have constructed a variety of vectors that contained the *lacF* gene as a selective marker under control of the replicon promoter of the lactococcal plasmid pSH71. Introduction of these homologous vectors into *L. lactis* NZ3000, which contains an in-frame deletion in the *lacF* gene, resulted in rapid growth and acid formation on lactose-containing medium, allowing for simple and efficient selection as well as stable maintenance of transformants.

The utility of the new series of cloning vectors carrying the *lacF* marker was shown by their transformation into expression plasmids that were equipped with the *lacA* promoter, which is known to be involved in efficient and moderately controlled transcription initiation in *L. lactis* (24). Moreover, in some expression vectors the transcriptional terminator of the lactococcal *pepN* gene was inserted to prevent transcription into the pSH71 replication region, which in some cases resulted in increased stabilities of the vector and the cloned DNA, as also observed for streptococcal DNA in *E. coli* (4). Finally, the expression vectors were equipped with a polylinker containing multiple unique cloning sites, allowing easy and efficient cloning.

All vectors carrying the *lacF* marker showed high segregational stability in *L. lactis* NZ3000 when grown on lactose-containing medium, as a consequence of the nature of the complementation system. This allows for their effective use in industrial applications, because many food fermentations are based on whey-derived media that contain lactose as the sole energy source. In addition, no apparent physical alterations were observed in the vectors even after prolonged growth on lactose-containing medium, indicating that chromosomal integration is an infrequent event or is not selected for. The region of homology between the vectors carrying the *lacF* marker and the chromosome of *L. lactis* NZ3000 is less than 0.3 kb. It is has been established that the recombination frequency in *L. lactis* is inversely related to the size of the region of homology and is more than 1,000-fold reduced when the region of homology is reduced from 3 to 0.3 kb (2). Remarkably, many of the vectors were also stable on glucose-containing medium. Most probably this is a consequence of their high copy numbers, which are characteristic of plasmids based on the pSH71 replicon (9, 10). The general applicability of the lactococcal *lacF* complementation system has recently been demonstrated by the construction of a food-grade vector, designated pF1846, based on the pSH71 replicon, which was also stably maintained under lactose selection (17). However, pF1846 still contained the transcriptional terminator of pSH71 and hence needed an additional promoter to drive expression of the *lacF* gene, limiting its application as an expression vector.

The application potentials of the expression vectors pNZ2106 and pNZ2107 have been tested by cloning the promoterless *E. coli gusA* gene under control of the *lacA* promoter and then analyzing its expression by blue-white colony screening of *L. lactis* and determining β-glucuronidase activities in lysates (18). These experiments showed the utility of the *pepN* terminator in pNZ2107, since with this vector the *gusA* gene was readily cloned and expressed, while with pNZ2106, lacking this terminator, only a single blue colony was obtained among 500 transformants. This suggests that most ligation products obtained with pNZ2106 were structurally unstable. Similar structural instability was observed during cloning of the *gusA* gene in the chloramphenicol resistance vector pNZ2103, illustrating the need for evaluating the application potentials of newly constructed vectors. While structural instability during gene cloning in *L. lactis* and other lactic acid bacteria has not been reported frequently (10), it has been observed in several cases, especially when the strong *lacA* promoter was used on high-copy-number plasmids (11, 18, 24). Therefore, this instability was studied in more detail by the characterization of pNZ2118, the plasmid found in the single blue colony obtained during the cloning of the *gusA* gene in pNZ2106. Sequence analysis of the *lacA* promoter region of pNZ2118 showed it to contain a mutation in the *lacA* promoter that involved the first thymidine residue of the TATAAT box (actual position, −11), which was replaced by a cytosine (Fig. 4). Although a systematic mutation analysis of the lactococcal promoter sequences has yet to be reported, this mutation is expected to reduce the promoter activity, since the first thymidine of this canonical −10 region is highly conserved in *L. lactis* promoters (10). It is possible that the mutated *lacA* promoter retains some residual activity that, in conjunction with the high copy number of pNZ2118, is responsible for the low level of *gusA* expression. Alternatively, it is possible that the residual *gusA* expression of pNZ2118 is due to read-through from the *repC* promoter, which also drives expression of the *lacF* gene. The latter possibility would also explain why *L. lactis* NZ3000 harboring pNZ2118 shows constitutive β-glucuronidase production (Table 2). However, footprint studies have shown that the LacR repressor protects the −31 to +6 region of the *lacA* promoter from DNase I digestion (26), and hence the mutation at position −11 may not only affect promoter efficiency but also result

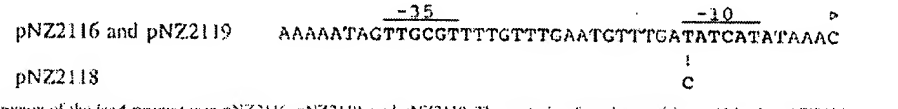

FIG. 4. Promoter region of the *lacA* promoter in pNZ2116, pNZ2119, and pNZ2118. The mutation found at position −11 in the pNZ2118 promoter is indicated, as are the −35 and −10 regions and the transcription initiation start site (arrowhead).

in a reduced binding of the LacR repressor, leading to the observed noninducible phenotype.

The most versatile expression vectors carrying the *lacF* marker that were constructed are the high-copy-number, stable plasmid pNZ2107 and its polylinker-containing derivatives pNZ2122 and pNZ2123, which contain the *lacA* promoter and the *pepN* terminator. When pNZ2107 is equipped with the *gusA* gene, such as in pNZ2119, and introduced in *L. lactis* NZ3000, it specifies a high level of β-glucuronidase activity that is induced twofold by growth on lactose compared with growth on glucose. Under full inducing conditions, pNZ2119 shows a level of β-glucuronidase activity approximately 10-fold-higher than that encoded by a previously constructed plasmid, pNZ276, consisting of pNZ124 carrying the *lacR* gene and *lacA-gusA* promoter fusion (18). This difference can be partly explained by the absence in pNZ2107 of the *lacR* repressor gene, which when present, such as in pNZ276, is known to reduce the efficiency of the *lacA* promoter but to increase the repression of this promoter in multicopy plasmids by preventing titration of the chromosomally encoded LacR repressor (24, 25). However, it cannot be excluded that the high and twofold-controlled *gusA* expression level is due to additional transcriptional read-through from the *repC* promoter and possibly to stabilization of the transcript as a result of the presence of the *pepN* terminator.

In this work we have described the construction and evaluation of a stable and convenient lactococcal food-grade cloning and expression system based on *lacF* complementation. Several of these vectors have been used for the cloning and overexpression of lactococcal genes in *L. lactis* (10, 11). The nucleotide sequences of all of the developed vectors are known, and they consist entirely of *L. lactis* DNA, indicating that they can be used for the improvement of lactococci by self-cloning, the simplest form of genetic modification that employs only homologous DNA and has a distinct regulatory status (10). This will allow for the further development of lactococci as acceptable hosts for the production of proteins, peptides, or metabolites for the food industry.

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